Cultured Human Melanocytes from Black and White Donors Have Different Sunlight and Ultraviolet A Radiation Sensitivities

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Short-term and long-term survival of cultured neonatal foreskin melanocytes from black and white individuals were assessed following a single exposure to simulated sunlight or ultraviolet A (UVA) radiation. Melanocytes from black individuals contained significantly more melanin than melanocytes from white individuals ($p < 0.05$). Black and white melanocytes had similar survival profiles following simulated sunlight exposure, whereas black melanocytes were significantly more resistant to UVA cytotoxicity than melanocytes from white subjects ($p < 0.05$) at UVA doses above 15 J/cm^2. There was no difference in unscheduled DNA synthesis in the black or white melanocytes following simulated sunlight exposure and no unscheduled DNA synthesis was measurable following melanocyte exposure to UVA radiation. Low-dose UVA (1 or 5 J/cm^2) was mitogenic to both black and white melanocytes. By analysis of co-variance, the melanin content of melanocytes of black and white subjects was significantly ($p < 0.05$) associated with susceptibility to UVA killing; melanocytes with high melanin content had high resistance to UVA cytotoxicity and those with low melanin content had low resistance to UVA cytotoxicity. From these data we suggest that the higher melanin content of melanocytes of black subjects confers increased resistance to UVA damage. This is likely to be of importance in epidermal photodamage. *J Invest Dermatol 99:454–459, 1992*

The greater susceptibility of white versus black skin to ultraviolet radiation (UVR)–induced photodamage is believed to be due to the inferior ultraviolet radiation filtering capacity of white skin [1]. Epidermis of black individuals is a superior UVR filter because it contains greater amounts of melanin than white epidermis. Kitano and Hu reported that pigmented B16 melanoma cells were more resistant to UVB cytotoxicity than non-pigmented B16 melanoma cells [2]. To our knowledge, no follow-up studies were performed comparing resistance of pigmented versus non-pigmented melanoma cells to UVB and UVA radiation. However, Westerhof et al [3] demonstrated in a subject with vitiligo that pigmented skin required twice the 310-nm UV energy to elicit erythema compared to vitiliginous skin. Additionally, pigmented skin required four times the 405-nm UV energy to elicit erythema than the vitiliginous skin. From these reports, it is evident that epidermis containing melanin pigment is effectively protected from damage from UVB exposure and highly effectively protected from UVA radiation. Because of these reported differences in the efficiency of melanin protection from UVB versus UVA radiation, we were interested in determining if the melanin content of human epidermal melanocytes would affect their resistance to damage by sunlight or UVA radiation. We chose melanocytes from black and white individuals as a model of epidermal cells containing different amounts of melanin. We measured melanin content of the cells, exposed the cells to simulated sunlight or UVA radiation, and then measured melanocyte survival and unscheduled DNA synthesis.

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Abbreviations:

EDTA: ethylenediaminetetraacetic acid
FCS: fetal calf serum
KGM: keratinocyte growth medium
MGM: melanocyte growth medium
PBS: phosphate-buffered saline
TCA: trichloroacetic acid
UVA: ultraviolet A
UVB: ultraviolet B
UVR: ultraviolet radiation

MATERIALS AND METHODS

Materials Primary cell cultures from neonatal foreskins were grown in keratinocyte growth medium (KGM, Clonetics Inc), which consisted of modified MCDB 153 with epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), calcium chloride (0.15 mM), bovine pituitary extract (0.4% v/v), and antibiotics (gentamicin and amphotericin B). Melanocyte growth medium (MGM) consisted of Ham's F-10 (Gibco, Inc., Grand Island, NY) supplemented with $NaHCO_2$ (1.2 g/l), HEPES (5.96 g/l), cholera toxin (2.5 nM), 12-0-tetradecanoylphorbol 13-acetate (TPA, 20 ng/ml), isobutyl methylxanthine (IBMX,100 nM) and calcium chloride (2.0 mM) purchased from Sigma Chemical (St. Louis, MO), and penicillin/streptomycin/Fungizone and 5% fetal calf serum (FCS) purchased from Irvine Scientific (Santa Anna, CA). Plastic tissue culture flasks were obtained from Becton Dickinson Labware, Lincoln Park, NJ. Trypsin, geneticin, sodium hydroxide, hydroxyurea, melanin (isolated from *sepia officinalis*), and bovine serum albumin (BSA, type V) were purchased from Sigma Chemical Co. and Dulbecco's phosphate-buffered saline (PBS) and 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution were ob-

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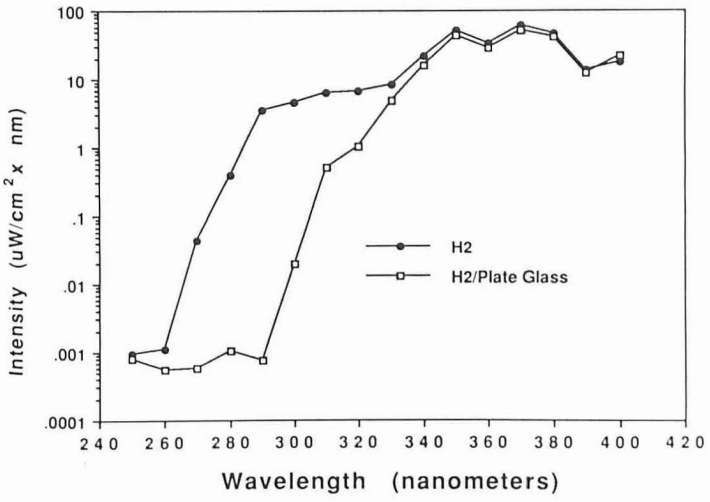

Figure 1. Spectral irradiance of the Dermalight 2001 solar simulator with an H2 filter (●) or with an H2 filter plus a sheet of 4-mm–thick plate glass (□).

tained from Irvine Scientific. Trichloroacetic acid and perchloric acid were obtained from Fisher Scientific.

Cell Culture Foreskins were visually sorted into black and white groups. Foreskins that were of intermediate, tan to gray coloration, were excluded from these experiments. Neonatal foreskin melanocytes were grown as previously described [4]. Briefly, keratinocytes and melanocytes from each individual donor were grown together in primary culture utilizing KGM as the culture medium. The cells were incubated in a 37°C, 5% CO_2 humidified environment and the culture medium was changed twice weekly. At confluence, primary cultures were separated by differential trypsinization, with keratinocytes passaged to new plates with KGM and melanocytes passaged to new plates with MGM. Fibroblast contamination of melanocyte cultures was eliminated by adding geneticin 100 μg/ml to each flask for the first 2 d after passage. Melanocytes were further passaged 1:2 when 80% confluent and confirmed to be of melanocyte lineage by their ability to oxidize 3,4-dihydroxyphenylalanine to melanin and by a positive reaction with anti-S100 antibodies [5].

Second- or third-passage melanocytes were used for these experiments. Cells were detached from culture plates with trypsin/EDTA solution, pelleted, resuspended in MGM, then seeded 5.0×10^4 cells per well onto 6-well plates for cell-survival experiments and 2.0×10^5 cells per well on 6-well plates for unscheduled DNA synthesis and pigment measurements. Cells were allowed to adhere to cell-culture plates for 48 h prior to being subjected to experimental conditions.

Melanocyte Irradiation Melanocytes were irradiated with a Dermalight 2001 Sol 3 Solar Simulator (Dermalight Systems, Studio City, CA) equipped with an H2 filter that excludes wavelengths below 280 nm. This high-pressure metal halide source mimics the spectrum of light emitted by the sun. Spectral output of the UVR source was confirmed (Fig 1) with an International Light IL1700/760D/791 monochronometer/radiometer (International Light, Newburyport, MA). During melanocyte-simulated sunlight irradiation, output of the source was measured with an International Light 1350 Radiometer utilizing an International Light UVB detector with peak sensitivity at 290 nm. UVA radiation was monitored with the same radiometer utilizing an International Light UVA detector with peak sensitivity at 360 nm. Ultraviolet light dosages were calculated depending on the measured output of the lamp. For simulated sunlight experiments, the fluence rate was 1.5 mW/cm² at 15 cm and for the UVA experiments the fluence rate was 20.0 mW/cm² at 15 cm. For the UVA experiments, a 4-mm–thick sheet of plate glass was used to exclude wavelengths below 320 nm [6]. As shown in Fig 1, the plate glass effectively filtered, by at least one decade, all wavelengths below 320 nm. Dose-response experiments were performed with melanocytes receiving 0, 5, 10, 20, 40, 80, 120, 160, 180, or 200 mJ/cm² simulated sunlight and 0, 1, 5, 10, 15, 20, 25, 30, or 40 J/cm² UVA radiation. Because of the heat generated by the UVR source, cells were irradiated with the culture plates on ice. Sham-irradiated cells were treated identically as the irradiated cells except that during the sham irradiation the plates were shielded from the UVR by a cardboard cover.

Prior to irradiation, culture medium was removed and the cells washed with PBS and then covered with a thin layer of PBS to prevent cell desiccation during irradiation. Following UVR exposure, the PBS was removed and the culture medium replaced. Three replicates were utilized for each ultraviolet dosage and controls.

Melanin Measurement On the day of irradiation, the cells were trypsinized and harvested, counted by hemacytometer, and centrifuged and the cell pellet dissolved in 1 N sodium hydroxide [7]. Absorbance of 410-nm light by melanocyte melanin was measured with a Milton Roy Spectronic 601 spectrophotometer. Melanocyte melanin content was then determined by comparing melanocyte melanin absorbance against a standard absorbance curve for Sigma melanin and expressed as micrograms melanin per 10^5 cells.

Cell Counts Melanocytes, when seeded onto culture plates, do not form colonies but instead spread out across the culture plates. Therefore, standard colony-forming assays [8] could not be performed. To assess melanocyte short-term survival following UVR exposure, cells were counted at 24 h after irradiation and, for long-term survival, cells were counted at 7 and 14 d. Prior to cell counts, to remove any nonviable cells, the plates were rinsed twice with PBS. The cells were then harvested by trypsinization and counted with a Coulter Counter (attenuation 0.707, aperture 8) and by hemacytometer with trypan blue, which confirmed cell viability of greater than 95%. Melanocyte percent survival at each time point was calculated as follows: ([viable cell number (irradiated)]/[viable cell number (unirradiated)]) × 100.

Unscheduled DNA Synthesis To inhibit melanocyte scheduled DNA synthesis, the irradiation group and sham-irradiation group cells were treated with 10 mM hydroxyurea for 12 h prior to irradiation. Immediately following UV exposure, the irradiated and sham-irradiated cells were pulsed with 0.1 μCi [^{3}H]-thymidine (6.7 Ci/mMole) for 6 h. Following the pulse, the cells were washed twice with PBS, harvested by trypsinization, counted with a hemacytometer, and processed for scintillation counting by the method of Kragballe et al [9]. Briefly, the DNA of the trypsinized cells was precipitated with 6% trichloroacetic acid (TCA) and the precipitate washed twice with 6% TCA. After centrifugation, the supernatants were saved for counting the [^{3}H]-thymidine acid soluble fraction. The precipitate was hydrolyzed with 3% perchloric acid at 95°C for 15 min and centrifuged (6000 × g for 10 min). [^{3}H]-thymidine incorporation into DNA was determined by counting the supernatant on a Beckman LS 7500 scintillation counter and expressed as counts per minute (CPM)/10^5 cells. The amount of unscheduled DNA synthesis (expressed as percent control) was then determined by the following formula: [(CPM/10^5 cells UV irradiated)/(CPM/10^5 cells sham UV irradiated)] × 100.

Statistical Analysis Statistical differences in melanocyte melanin content and unscheduled DNA synthesis between the black and white groups were determined by Student t test. Analysis of covariance was employed to determine the effects of melanin content and cell type on survival following melanocyte UVR exposure. The statistical software programs STATWORKS (Cricket Software Inc, Philadelphia, PA) and JUMP (SAS Institute, Cary, NC) were utilized for the calculations. Statistical significance was defined as $p < 0.05$.

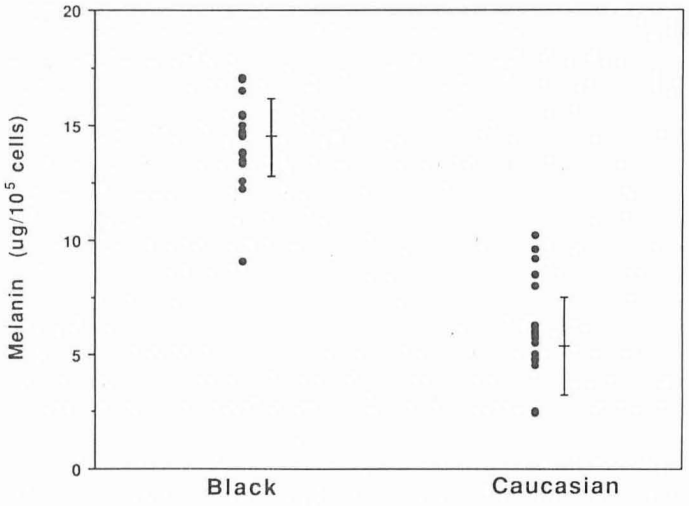

Figure 2. Melanin content of melanocytes from black and white individuals. Each *point* represents the melanin content of melanocytes from an individual donor (black, n = 25; white, n = 25). *Horizontal bars,* mean of each data set; *vertical bars,* standard error of the mean.

RESULTS

As predicted, melanocytes from black individuals had significantly greater ($p < 0.05$) melanin content than white (Fig 2).

Human neonatal foreskin melanocytes from black and white donors had equivalent 24-h survival following a single exposure of simulated sunlight (290–400 nm) (Fig 3*A*). Similarly, the 7-d and 14-d long-term survival profiles of melanocytes from black and white subjects were nearly identical (Fig 3*B*,*C*).

Melanocytes from white subjects had significantly increased cytotoxicity ($p < 0.05$) compared to melanocytes from black subjects at UVA doses greater than 15 J/cm² (Fig 4*A*,*B*,*C*). Decreased survival of melanocytes from white subjects was observed at 24 h and became even more evident at 7 and 14 d. By analysis of co-variance, the melanin content of melanocytes from black and white subjects was significantly ($p < 0.05$) associated with susceptibility UVA killing — melanocytes with high melanin content had high resistance to UVA cytotoxicity and those with low melanin content had low resistance to UVA cytotoxicity.

No difference in DNA repair was noted between the cell types following simulated sunlight exposure (Fig 5) and no measurable DNA repair was found in either cell type following UVA irradiation (data not shown).

Low-dose UVA (1 and 5 J/cm²) exposure caused a significant increase in melanocyte proliferation in black and white subjects compared to unirradiated controls ($p < 0.05$, Fig 4*B*,*C*). The mitogenic effect was best demonstrated by expressing the data on the ordinate in a linear format rather than a logarithmic format. Therefore all the graphs were prepared in a linear format. Although an increase in melanocyte numbers was noted following low-dose simulated sunlight exposure, the increase was not significant ($p > 0.05$, Fig 3*B*,*C*).

DISCUSSION

We have demonstrated that melanocytes from black and white subjects, despite significant differences in melanin content, were equally resistant to the cytotoxic effects of a single exposure to simulated sunlight wherein the highest photon energy is in the UVB range. Conversely, melanocytes from white subjects were more susceptible to the cytotoxic effects of UVA radiation than melanocytes from black subjects.

Distinct mechanisms by which the different UVR spectra injure and kill cells can be implied from the cell-survival curves. The simulated sunlight 24-h survival curve is almost horizontal, with no

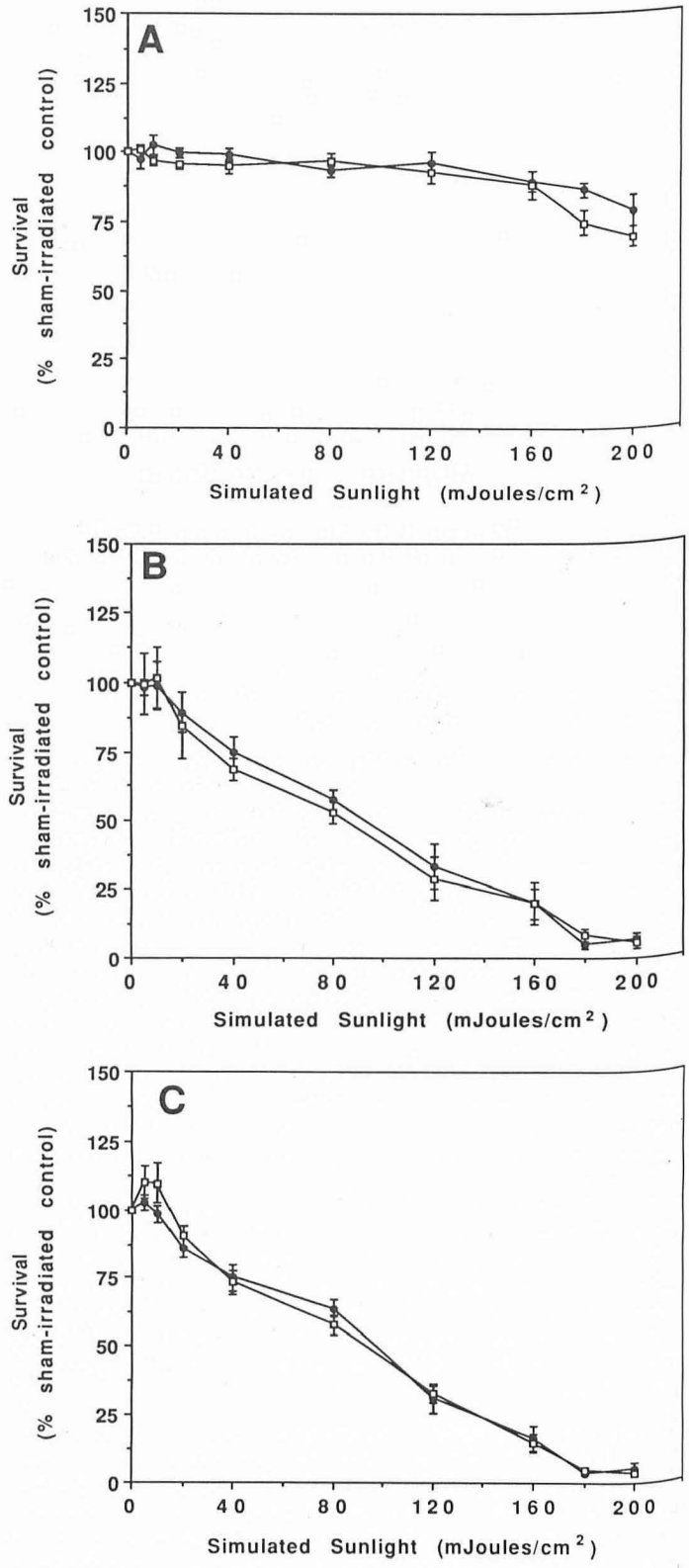

Figure 3. Human melanocyte survival following exposure to simulated sunlight. Melanocytes from nine black donors (●) and nine white donors (□) were tested. Increasing doses of simulated sunlight (mJ/cm²) are labeled along the abscissa and the surviving fraction of cells (% sham-irradiated control) is along the ordinate. Twenty-four hour *(A)*, 7 d *(B)*, and 14 d *(C)* survival following exposure to simulated sunlight. Each data *point* represents the mean ± SEM survival of all the donors tested for that dose of UVR. The range of cell counts for the sham-irradiated controls were 24-hour black, $2.2–4.8 \times 10^4$ and white, $2.4–5.0 \times 10^4$; 7 d black, $5.7–12.4 \times 10^4$ and white, $4.5–12.7 \times 10^4$; 14 d black, $10.9–31.0 \times 10^4$ and white, $10.8–32.6 \times 10^4$.

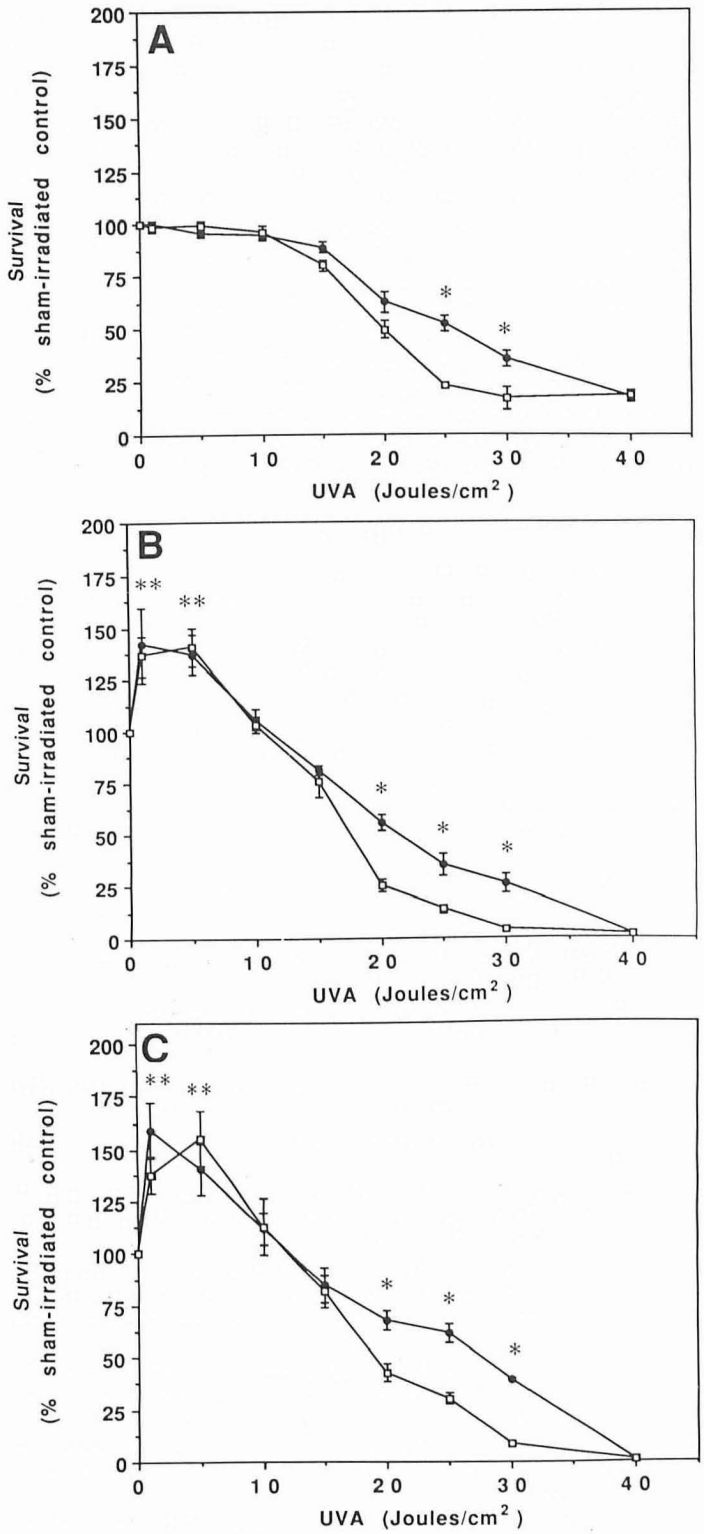

Figure 4. Human melanocyte survival following exposure to UVA radiation. Melanocytes from ten black donors (●) and ten white donors (□) were tested. Increasing doses of UVA radiation (J/cm²) are labeled along the abscissa and the surviving fraction of cells (% sham-irradiated control) is along the ordinate. Twenty-four hour *(A)*, 7 d *(B)*, and 14 d *(C)* survival following exposure to UVA radiation. Each data *point* represents the mean ± SEM survival of all the donors tested for that dose of UVR. The range of cell counts for the sham-irradiated controls were 24 hour black, $2.2-4.8 \times 10^4$ and white, $2.5-4.9 \times 10^4$; 7 d black, $5.2-12.3 \times 10^4$ and white, $6.2-11.0 \times 10^4$; 14 d black, $10.2-30.5 \times 10^4$ and white $9.3-30.0 \times 10^4$.* Black versus white melanocyte survival, $p < 0.05$.** Mitogenic effect of low-dose UVA (UVA-irradiated black and white melanocytes versus sham-irradiated controls, $p < 0.05$).

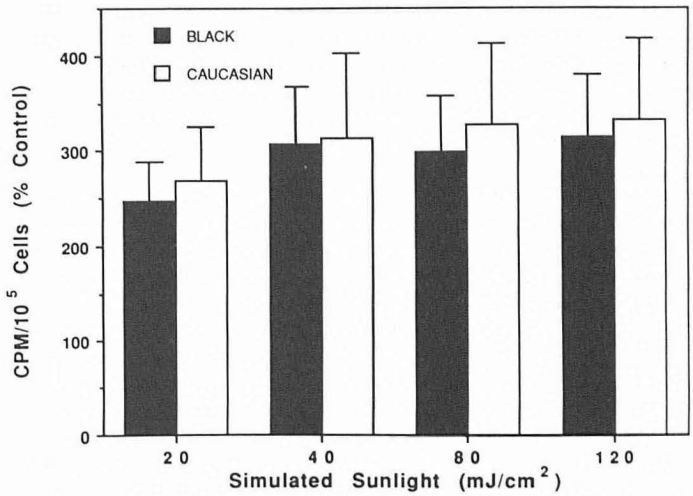

Figure 5. Unscheduled DNA synthesis in human melanocytes from black (n = 6) and white (n = 6) donors following simulated sunlight exposure. Each donor's melanocytes were split into irradiation (20, 40, 80, and 120 mJ) and sham-irradiation sets. Immediately following irradiation or sham irradiation the cells were pulsed with [³H]-thymidine. Unscheduled DNA synthesis was measured at 6 h post-irradiation. The sham-irradiated control absolute counts for each individual donor were comparable, approximately $3-5 \times 10^2$ counts per 10^5 cells. The results are expressed as percent sham-irradiated control ± SEM.

significant decrease in cell number, except at the highest doses of UVR. The full extent of simulated sunlight-induced cytotoxicity was noted at 7 d. In contrast, at doses above 15 J/cm², UVA lethal damage was readily apparent at 24 h. Except at low UVA doses, the 24-h, 7-d, and 14-d survival curves are comparable. UVA-induced cell damage is likely to have been widespread and of such a high degree that the cells were unable to repair the damage and died within 24 h. One possible mechanism is UVA-induced generation of melanin free radicals [10] and/or oxygen free radicals such as superoxide [11] and lipid hydroperoxides [12]. Reactive species damage to DNA or to other cellular systems such as membranes, structural proteins and enzymes may have produced sufficient injury to exceed the reparative mechanisms in the cell culminating in cell death. In contrast, melanocytes exposed to simulated sunlight sustained a type of injury that required more time to become evident. Although not proved in these experiments, the cells exposed to simulated sunlight may have died when attempting mitosis. This phenomenon has been demonstrated in cells exposed to ionizing radiation [13]. The DNA damage incurred by simulated sunlight exposure may have been insufficient to cause immediate cytotoxicity but became apparent when cells attempted new DNA synthesis and mitosis. Because of the slow population doubling time of melanocytes, this reproductive failure [13] would have taken several days for enough cells to die while attempting mitosis to manifest as a significant decrease in cell numbers.

No difference in unscheduled DNA synthesis was found between the cell types following simulated sunlight exposure. From these data we believe that melanocytes from both black and white subjects sustained equivalent amounts of DNA damage from simulated sunlight. Our unscheduled DNA synthesis results are consistent with those of Ishikawa et al [14], who reported no difference in unscheduled DNA synthesis in combined UVB/UVA irradiated guinea pig nonpigmented and pigmented skin in vivo. A recent study by Schothorst et al [15] comparing the amount of DNA damage and repair in human keratinocytes from white subjects and melanocytes following exposure to UVB radiation found that melanocytes sustained equal or greater numbers of thymine dimers than keratinocytes. Pigmentation did not afford any increased protection of melanocytes from pyrimidine dimer formation. The perinuclear location

of melanosomes in black individuals may play a pivotal role in DNA protection from UVR injury. Melanocytes from black subjects grown in vitro may not have a perinuclear melanosomal arrangement that could make them equally susceptible to DNA damage by simulated sunlight. These reports and our data raise interesting questions as to the role of melanin in protecting DNA from UVB damage.

The unmeasurable DNA repair activity in the UVA-irradiated melanocytes may have been due either to negligible UVA-induced DNA damage or inactivation of DNA repair enzymes by UVA or UVA-induced reactive species. Sutherland et al [16] were able to detect *Micrococcus luteus* endonuclease-sensitive sites in DNA from human skin irradiated in vivo with high-dose UVA (50–150 J/cm^2). However, keratinocytes were the predominant cell type in their assays and they did not evaluate unscheduled DNA synthesis in the skin of their irradiated subjects. UVA radiation has also been found to cause DNA single-strand breaks and the formation of DNA-protein crosslinks but the biologic significance of these lesions is not clear [17]. Further work is necessary in this area to clarify the effects of UVA radiation on DNA damage and DNA repair.

Melanocyte exposure to 1 or 5 J/cm^2 UVA profoundly increased the mitotic activity of the cells compared to the sham-irradiated controls ($p < 0.05$). This effect was notable at 7 d after UVA exposure and became even more pronounced at 14 d. We do not believe the significant increase in mitotic activity can be explained solely by mitotic delay [13], as the mitotic index returns to pre-irradiation values after radiation exposure. Low-dose UVA apparently induces mitogenic stimuli that result in substantially increased melanocyte cell numbers. UVA-induced reactive oxygen species may be one of the yet to be characterized mitogenic stimuli. In other systems the generation of intracellular superoxide is associated with tumor promotion [18]. Simulated sunlight had no significant mitogenic effect on human melanocytes in these experiments. Our results differ from those of Libow et al who reported that 2.5 mJ/cm^2 UVR (270–360 nm) was mitogenic to cultured human melanocytes [19].

The discrepancy in survival between melanocytes from black and white subjects following UVA exposure may not be exclusively due to greater amounts of melanin in melanocytes from black patients but also due to the type of melanin present in the cells. Melanin acts as a filter by absorbing and reflecting UVR [20]. In addition to its filtering ability, melanin also acts as a scavenger of UVR-induced reactive species [21,22]. However, not all types of melanin are equally able to effectively and safely handle reactive species. It has been amply demonstrated that pheomelanin is photosensitizing when exposed to UVA radiation and can enhance UVR phototoxicity by the production of excess reactive species [10,23,24]. Pheomelanin is the type of melanin produced by most melanocytes in white people, and is found either in low concentrations or not at all in melanocytes from black individuals [25]. Eumelanin, the type of melanin found predominantly in the epidermis of black individuals, is better able to neutralize ROS and does not produce excess reactive species following UVR exposure [26].

Recently, we determined that neonatal foreskin melanocytes from black and white donors possess similar catalase, glutathione peroxidase, and superoxide dismutase activities [27]. Moreover, the melanocyte antioxidant enzyme activity levels were lower than neonatal foreskin keratinocytes and fibroblasts. This confirmed previous work by Norris et al who demonstrated that human melanocytes were highly susceptible to cytotoxicity by hydrogen peroxide and that human keratinocytes and fibroblasts were more resistent to hydrogen peroxide cytotoxicity than melanocytes [5]. This further substantiated the theory that lower antioxidant enzyme activity of melanocytes may affect their resistance to UVR-induced reactive species. Melanocytes from white people, which do not possess greater antioxidant enzyme activity levels than melanocytes from black people, and contain potentially phototoxic pheomelanin may, following UVA exposure, generate excess reactive species that exceed their antioxidant defenses, culminating in cell injury and death.

Although high doses of UVA were required to produce the cytotoxic effects, these experiments utilized only single exposures of UVR. Moreover, UVA is longer wavelength radiation than UVB and penetrates deeper into the epidermis. Whereas most UVB is filtered by the stratum corneum and malpighian layers of the epidermis [1,28], UVA penetrates to the basal layer where melanocytes and basal keratinocytes reside. Multiple low-dose exposures of the more deeply penetrating UVA may have significant effects on the epidermis. Further experiments are necessary to explore multiple-dose effects of UVA on epidermal cells. Regardless, we believe that this is additional evidence of the harmful effects of UVA radiation. UVA-mediated skin damage is likely to be critical in those individuals who use sunscreens that do not block UVA and those individuals who expose their skin to high doses of UVA in tanning booths. High doses of UVA in a single exposure is possible in these situations. Repeated UVA damage over an extended period of time to basal keratinocytes and melanocytes is likely to be an additional mechanism in photodamage to the epidermis [29].

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